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CERTIFICATE

This certificate is issued in support of an application for Patent registration in a country outside New Zealand pursuant to the Patents Act 1953 and the Regulations thereunder.

I hereby certify that annexed is a true copy of the Provisional Specification as filed on 18 September 2003 with an application for Letters Patent number 528323 made by JOHN STANTON MITCHELL and YINQIU WU.

Dated 1 October 2004.

Neville Harris
Commissioner of Patents, Trade Marks and Designs

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PROVISIONAL SPECIFICATION

IMMUNOASSAY

We, YINQIU WU, a New Zealand citizen of 76 Hudson Street, Hillcrest, Hamilton, New Zealand, and JOHN STANTON MITCHELL, a New Zealand citizen of 24 Basley Road, Rotorua, New Zealand, do hereby declare this invention to be described in the following statement:

Immunoassay

FIELD OF THE INVENTION

The present invention relates to a method for a competitive determination of haptens, more particularly small molecular weight haptens using an immunoassay format.

BACKGROUND

In sandwich or "catching antibody---antigen---labelled antibody" assays, two independent epitopes bound by different antibodies provide the advantages in terms of speed, sensitivity, and specificity. However, sandwich assay formats have not been directly applicable to small molecular weight haptens,. Haptens are not large enough to bind simultaneously to two antibodies independently. For these reasons, competitive assays are the most widely used format for measurement of haptens.

To enhance assay sensitivities and specificities for haptens, non-competitive methods have been used. For example, *anti*-immune complex assays (*Proc. Natl. Acad. Sci. USA*, **90**, 1993, 1184-1189 and *Clin. Chem.* **40**(11), 1994, 2035-2041) were successfully used for determinations of tetrahydrocannabinol (THC) and digoxin. Selective antibody or 'idiometric' methodology (*J Immunol Methods* **181**, 1995, 83-90 and *Steroids* **60**, 1995, 824-829) is another non-competitive approach, which provided more sensitive assays for estradiol and progesterone than conventional competitive enzyme assays. However, these non-competitive formats require unique antibodies and antiidiotypes that are potentially difficult to obtain. Another non-competitive two-site enzyme immunoassay format (hetero-two-site or immune complex transfer) (*Biotechnology Annual Review* 1,1995, 403-451) has been also applied for small peptides or haptens with good detection levels. Unfortunately the immunoassay requires multiple steps. Multiple steps mean the assay is generally more expensive and time consuming than is desirable. The immunoassay also involves the use of harsh chemicals which potentially damage sensitive biomolecules and also involve the use of strongly acidic, basic or organic solvents that complicate providing assays in non-laboratory settings.

Another non-competitive assay for small molecules has been employed for measurement of cortisol and estradiol as described in US 6,037,185. This assay permits the direct measurement of hapten bound sites or initial amount of hapten in the sample. Unfortunately, the assay still requires multiple steps to perform, which is potentially costly and time consuming.

Optical immunosensors are popular for bio-analysis. The non-destructive nature of the technology permits multiple reuse of samples for other readings. Rapid signal generation and thus rapid result generation are also advantages of the system. Unfortunately, label-free optical immunosensors have relatively poor analytical sensitivities to haptens with low molecular weight compared to traditional large molecule immunoassays. Despite significant developments in this field, optical immunosensors tend to be one magnitude less sensitive than commercial immunoassays for determining haptens. This problem is particularly acute when detecting low molecular weight haptens.

It is an object of the present invention to provide an immunoassay that overcomes at least some of the above-mentioned disadvantages of existing assays; and/or that provides similar or better sensitivities to those of existing non-competitive formats; and/or that is rapid; and/or that has fewer steps than assays in the art, or that at least provides the public with a useful choice.

SUMMARY OF THE INVENTION

In a first aspect, the present invention provides a method for detecting a hapten in a sample comprising the steps of:

a) providing a sample potentially containing a hapten of interest;
b) contacting the sample with a pre-determined amount of a first moiety, said first moiety being bound to a signaller and separated therefrom by a first linker, which first moiety is either:
    i. a binding partner that specifically binds to the hapten of interest, or
    ii. the hapten of interest or an analogue thereof;

c) contacting the resultant mixture of a) and b) with an immobilised second moiety, said second moiety being bound to an immobilisation substrate and separated therefrom by a second linker, which second moiety is either:

   i. a binding partner that specifically binds to the hapten of interest, or

   ii. is the hapten of interest or an analogue thereof,

   providing that when the first moiety is a binding partner, the second moiety is a hapten or hapten analogue and when the first moiety is a hapten or hapten analogue, the second moiety is a binding partner; and

d) detecting the amount of first moiety bound to second moiety.

In a further aspect, the present invention provides a method for detecting a hapten in a sample comprising the steps of:

a) providing a sample potentially containing a hapten of interest;

b) contacting the sample with a pre-determined amount of a binding partner that specifically binds to the hapten of interest, said binding partner being bound to a signaller and separated therefrom by a first linker;

c) contacting the resultant mixture of a) and b) with an immobilised hapten of interest or an analogue thereof, said hapten or analogue thereof being bound to an immobilisation substrate and separated therefrom by a second linker; and

d) detecting the amount of binding partner bound to said immobilised hapten or an analogue thereof.

In a still further aspect, the present invention provides a method for detecting a hapten in a sample comprising the steps of:

a) providing a sample potentially containing a hapten of interest;

b) contacting the sample with a pre-determined amount of hapten of interest or an analogue thereof, said hapten or analogue thereof being bound to a signaller and separated therefrom by a first linker;

c) contacting the resultant mixture of a) and b) with an immobilised binding partner that specifically binds to the hapten of interest, said binding partner being bound to an immobilisation substrate and separated therefrom by a second linker; and

d) detecting the amount of hapten or analogue thereof bound to said immobilised binding partner.

In a yet further aspect, the present invention provides a method for detecting a hapten in a sample comprising the steps of:

a) providing a sample potentially containing a hapten of interest;

b) contacting the sample with a pre-determined amount of a first moiety, said first moiety being bound to a signaller, which first moiety is either:

   i. a binding partner that specifically binds to the hapten of interest, or

   ii. the hapten of interest or an analogue thereof;

c) contacting the resultant mixture of a) and b) with an immobilised second moiety, said second moiety being bound to an immobilisation substrate, which second moiety is either:

   i. a binding partner that specifically binds to the hapten of interest, or

   ii. is the hapten of interest or an analogue thereof,

   providing that when the first moiety is a binding partner, the second moiety is a hapten or hapten analogue and when the first moiety is a hapten or hapten analogue, the second moiety is a binding partner; and

d) detecting the amount of first moiety bound to second moiety,

characterised in that said first moiety is bound to and separated from said signaller by a first linker and said second moiety is bound to and separated from said immobilisation substrate by a second linker.

In another aspect, the present invention provides a kit for determining the presence of a hapten of interest in a sample, which kit at least includes:

a) a first moiety being bound to a signaller and separated therefrom by a first linker, which first moiety is either:

   i. a binding partner that specifically binds to the hapten of interest, or

   ii. the hapten of interest or an analogue thereof; and

b) an immobilised second moiety, said second moiety being bound to an immobilisation substrate and separated therefrom by a second linker, which second moiety is either:

   i. a binding partner that specifically binds to the hapten of interest, or

   ii. is the hapten of interest or an analogue thereof,

providing that when the first moiety is a binding partner, the second moiety is a hapten or hapten analogue and when the first moiety is a hapten or hapten analogue, the second moiety is a binding partner.

In another aspect, the present invention provides a kit for determining the presence of a hapten of interest in a sample, which kit at least includes:

a) a first moiety being bound to a signaller, which first moiety is either:
   i. a binding partner that specifically binds to the hapten of interest, or
   ii. the hapten of interest or an analogue thereof; and
b) an immobilised second moiety, said second moiety being bound to an immobilisation substrate, which second moiety is either:
   i. a binding partner that specifically binds to the hapten of interest, or
   ii. is the hapten of interest or an analogue thereof,

   providing that when the first moiety is a binding partner, the second moiety is a hapten or hapten analogue and when the first moiety is a hapten or hapten analogue, the second moiety is a binding partner,

characterised in that said first moiety is bound to and separated from said signaller by a first linker and said second moiety is bound to and separated from said immobilisation substrate by a second linker.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows a rapid optical biosensor-based immunoassay format using "dual-linker design with nanoparticle enhancement".

FIG. 2 shows the structure of progesterone-ovalbumin conjugate with a 25-atoms linker (3), and its synthesis from the conjugate (1) (*Steroids,* **67,** 2002, 565-572). The conjugate (3) was immobilised onto the SPR biosensor surface.

FIG. 3 shows the structure of antibody-linker-nanogold conjugate (6) through the biotin/streptavidin linkage, and its preparation from commercial biotin agent BcapNHS (4) with monoclonal *anti*-progesterone antibody (B) and followed by reaction with commercial streptavidin-nanogold particles (10 nm).

FIG. 4 shows the standard curve (RU percentage value to RU at 0 progesterone concentration versus concentration of progesterone in the range 0 to 1 μg/ml measured according to the method of this invention.

FIG. 5 shows the chemical structures of steroids including estrogens, progesterone and some steroids having an A-ring structure similar to progesterone, and two neurotransmitter molecules or dopamine and serotonin.

FIG. 6 shows chemical structures of some hapten-linker molecules (steroids and neurotransmitter derivatives) having different end-functional groups.

FIG. 7 shows response values of antibody/nanogold in different volume ratio on the progesterone-linker (11-atoms)-OVA conjugate sensor surface

FIG. 8 shows response values of antibody/nanogold in different volume ratio on the progesterone-linker (25-atoms)-OVA conjugate sensor surface

FIG. 9 shows values of 50% bound and lowest detection limits for both Conjugates in progesterone assay

DETAILED DESCRIPTION OF THE INVENTION

As stated above, the present invention provides, in a first aspect, a method for detecting a hapten in a sample. The method comprises several essential steps.

The first step is providing a sample potentially containing a hapten of interest. The sample is then contacted with a pre-determined amount of a first moiety. The first moiety is provided bound to a signaller and separated therefrom by a first linker. The first moiety is either a binding partner that specifically binds to the hapten of interest or the hapten of interest or an analogue thereof.

The mixture of the above steps is now contacted with an immobilised second moiety. The second moiety is provided bound to an immobilisation substrate and separated therefrom by a second linker. The second moiety is either a binding partner that

specifically binds to the hapten of interest, or is the hapten of interest or an analogue thereof. However, when the first moiety is a binding partner, the second moiety must be a hapten or hapten analogue. Alternatively, when the first moiety is a hapten or hapten analogue, the second moiety must be a binding partner.
The amount of first moiety bound to second moiety is then detected.

In the context of this invention, the term "hapten" means any small molecular hapten which has a molecular weight less than 5000 Daltons. Most usually, the hapten is a low molecular weight organic compound that reacts specifically with an antibody and which is incapable of eliciting an immune response by itself but is immunogenic when complexed with an antigenic carrier. Haptens of interest here are selected from the group comprising carbohydrates, polynucleotides, steroids, steroid analogues, polypeptides (such as peptide hormones), drugs and toxins, but are not limited thereto. Haptens of particular interest in the present invention include therapeutic drugs, narcotics, steroids, thyroid hormones, metabolites and pollutants.

Herein, "binding partner" refers to macromolecules capable of specifically binding to a target hapten of interest. Examples of suitable macromolecules include antibodies and fragments thereof as well as nucleic acids, such as an RNA aptamer described in *Biochemical and Biophysical Research Communications* 281, 237-243 (2001) and incorporated herein by reference.

Antibodies are well known to those of ordinary skill in the science of immunology. As stated above, included within the ambit of "binding partner" are not only intact antibody molecules but also fragments of antibody molecules retaining hapten binding ability. Such fragments are also well known in the art and are regularly employed both *in vitro* and *in vivo*.

Therefore, "binding partner" also includes not only intact immunoglobulin molecules but also the well-known active fragments $F(ab')_2$, and Fab. $F(ab')_2$, Fab fragments which lack the Fc fragment of intact antibody, Fv, single chain (ScFv), mutants thereof, fusion proteins comprising an antibody portion, and any other modified configuration of the immunoglobulin molecule that comprises an antigen recognition

site of the required specificity. In an alternative embodiment, the binding partner may be a T-cell receptor.

"specifically binds" or "specifically binding" in the present invention means that the binding partner binds to the hapten of interest without substantial cross reactivity to other species in the sample to enable a meaningful detection result to be obtained.

"analogue" of a hapten herein refers to a group that competes with the hapten for binding to a binding partner. In the case of antibodies, the analogue should bind to the same site on the antibody as the hapten.

"sample" is typically a liquid sample from a biological source, but is not limited thereto.

"immobilisation substrate" is any bulky suitable substantially insoluble support that permits attachment of a linker. "immobilisation substrate" includes but is not limited to a chip surface, gels (e.g. cross-linked chromatography gels) and a solid support as well as any other support well known in the art. Non-limiting examples of suitable immobilisation substrates suitable for the practice of the present invention include:
(a) insoluble polymeric materials such as polystyrene, polypropylene, polyester, polyacrylonitrile, polyvinyl chloride, polyvinylidene, polysulfone, polyacrylamide, cellulose, cellulose nitrate, cross-linked dextrans, fluorinated resins, agarose, crosslinked agarose, and polysaccharides etc;
(b) glass, glass fibres, and glass beads;
(c) metal (gold, silver or platinum), metal strips and metal beads;
(d) nylon mesh material and nylon membranes; and
(e) test tubes, microtiter plates, dipsticks, lateral flow devices, resins, PVC, latex beads and nitrocellulose.

The invention is directed to "rapid" assays, characterised in that they are flow-through or flow-over assay formats, giving rapid signal generation and a reading typically in less than 10 minutes. The invention is particularly suited to a rapid flow-through assay using a commercial BIAcore instrument such as BIAcore 2000.

In one embodiment of the present invention, hapten molecules are chemically immobilised onto a sensor surface with a linker interposed between the hapten and the surface. In an alternative embodiment, the hapten is attached to an attachment intermediate material with a linker interposed between the hapten and the attachment intermediate material. The attachment intermediate is, in turn, attached to a sensor surface. Preferred attachment intermediates are selected from the group comprising proteins (*Steroids,* **67,** 2002, 565-572), nucleic acid fragments (US Patent: 5,849,480) and *N*-vinylpyrrolidone copolymer (US Patent: 5,723,334). Examples of suitable proteins as attachment intermediate materials include bovine serum albumin (BSA), ovalbumin (OVA) or keyhole limpet hemocyanin (KLH). Proteins may also include enzymes, secretory proteins, globular proteins. A preferred protein for use herein is ovalbumin (OVA). Where the protein is an enzyme, it is preferred that it be selected from the group comprising alkaline phosphatase, glucose oxidase, horseradish peroxidase and amine-enriched horseradish peroxidase.

Where the hapten is a steroid, it is preferred that binding of the hapten to the linker occurs at the 4-position of the A-ring structure. The binding at the 4-position of the A ring is particularly preferred when binding estrogens, progesterone and steroids having an A-ring structure similar to progesterone. Moieties illustrated in Figure **5** (other than Dopamine and Serotonin) are currently preferred steroids for binding at the 4-position on the A ring.

When the hapten is a neurotransmitter molecule such as dopamine or serotonin (as shown in Figure **5**), it is preferred that binding of the hapten occurs at the aromatic ring.

In the currently most preferred embodiment, the hapten is progesterone.

The "first linker" and "second linker" are typically each independently 4 to 50 atoms in length, preferably 10 to 50, more preferably 10 to 30 atoms in length excluding any bridging groups. Linkers suitable for the practice of the present invention are preferably (a) a carbon-based chain; (b) carbon-chain containing one or more heteroatoms such as N, S, O; (c) carbon-chain with substituted groups; (d) an amino acid chain, amino acid fragments incorporated into the chain, or multiple amino-acid

fragments chain by homologation; (e) a polyethylene glycol chain; (f) a chain have one or more sites of unsaturation such as alkenyl; (g) a nucleic acid chain; or (h) a polysaccharide chain etc. Obviously, depending on the nature and physical size of the moiety attached to the chain, the chain can be made hydrophobic or hydrophilic by including fewer or more groups respectively that are more polar or ionic in the chain.

The second linker can be selected from different molecular types and lengths. It has been found that the best performance is obtained when the second linker is selected to ensure that non-bulky groups are proximal the hapten. It is preferred that the chain be carbon-based. The carbon-based chain may comprise one or more heteroatoms selected from N, S, and O. Side chain substituent groups may also be provided. Other preferred chains are selected from the group comprising amino acids, a polyethylene glycol, alkyl, alkenyl, nucleic acid, and polysaccharide. The chain can have one or more sites of unsaturation. Multiple amino-acid fragments may be provided by homologation. The use of hybrid peptide-nucleic acid fragments as linkers is also contemplated.

A preferred synthesis of the first and second linkers for use in the present invention in different length is controlled and performed by successive aminocaproic acid homologation of hapten acid derivatives as illustrated in Figure 2 before conjugation to proteins or immobilised onto the sensor surface directly. Any suitable linker known in the art may be employed. Other examples of hapten-linker molecules useful in the practice of the present invention having different end-functional groups are shown in (Figure 6).

There are many well-known immobilisation techniques in the art. Preferred immobilisation techniques for immobilising the first moiety, hapten to be immobilised or binding partner to be immobilised onto a sensor surface is by a covalent coupling reaction (e.g. to an amine, a carboxyl or sulfhydryl group on the protein), nucleic acid hybridisation, or ligand interaction. Immobilisation on the sensor surface may be also by passive adsorption, or via a ligand interaction, such as an avidin/biotin complex (US Patent: 4,467,031).

In order to covalently bind hapten to first and second linking groups in the practice of the present invention, it is often necessary to include a thioether or ether bridging group, preferably a thioether group, generally through their mono-bromide intermediate compounds.

"signaller" herein means a group capable of providing an unambiguous indication to a detector of its presence. Preferred embodiments include nanoparticles, fluorescent markers, chromatic ions and complexes, metal or non-metal colour particles (such as immunogold and coloured latex beads), and groups incorporating one or more radioactive isotopes. Particularly preferred in the present invention are nanoparticles.

The term "nanoparticles" refers to the particles used to provide sensitivity through mass labels and are solid particles ranging widely in the size of nanoscale, which includes metal particles (colloidal gold), non-metal particles (latex beads), or any other suitable nanoparticles used as mass labels for signal enhancement.

Detecting the amount of bound double linker moieties of the present invention may be undertaken utilising a number of different techniques available in the art.

A particularly preferred detection system is the use of "optical biosensors". "optical biosensors" are a group of immunoassay instruments using visible radiation, optical waveguides and sophisticated optoelectronics as transducer techniques. The instrument used by the inventors includes but is not limited to surface plasmon resonance (SPR), total internal reflection spectroscopy (TIRS), ellipsometry and optical dielectric waveguides.

One having ordinary skill in the arts can detect protein binding using well-known methods. Various immunoassay procedures are described in *Immunoassays for the 80's*, A. Voller. *et al.*, Eds., University Park, 1981. These include enzyme detection systems, dyes (such as fluorescent markers, and chromatic ions and complexes) and metal or non-metal colour particles (such as immunogold and coloured latex beads), and incorporated radioactive isotopes (detectable by, for example, scintillation counting).

In one embodiment, immunogold particles are used because they are inexpensive and relatively stable. Suitable enzymes which may be used as a signaller include, but are not limited to, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase.

By radioactively labelling the signaller, it is possible to detect it through the use of a radioimmunoassay (RIA) (see, for example, Work, T. S., *et al.*, Laboratory Techniques and Biochemistry in Molecular Biology, North Holland Publishing Company, N.Y., 1978). The radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography. Isotopes which are particularly useful for the purpose of the present invention are: ^{3}H, ^{14}C, ^{35}S, ^{125}I and ^{131}I.

Fluorescent labels fall within the scope of the present invention. When a fluorescent-labelled signaller is exposed to light of the proper wavelength, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labelling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde, fluorescamine and bilirubin.

The signaller can also be detectably labelled using fluorescence-emitting metals such as ^{152}Eu, or others of the lanthanide series. These metals can be attached to the signaller using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

The signaller can also be a chemiluminescent group. The presence of the chemiluminescently labelled group is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labelling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound may be used as the signaller. Bioluminescence

is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Typically useful bioluminescent compounds for purposes of labelling are luciferin, luciferase and aequorin.

Detection of the signaller may be accomplished by a scintillation counter, for example, if the detectable label is a radioactive gamma emitter, or by a fluorometer, for example, if the label is a fluorescent material.

In the case of an enzyme label, the detection can be accomplished by colorimetric methods, which employ a substrate for the enzyme. Detection may also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

Where the protein is an enzyme, it is preferred that it be selected from the group comprising alkaline phosphatase, glucose oxidase, horseradish peroxidase and amine-enriched horseradish peroxidase.

The inventors have discovered that provision of a double linker molecule of the present invention increases binding partner binding performance in short-duration assays, such as flow-through assays leading to better assay sensitivities than with single linker or no linker systems. It has also been discovered that a most preferred detection system, surface plasmon resonance (SPR) utilising nano-particles gives unexpectedly good sensitivities when used in conjunction with double linker technologies.

It has also been found by the inventors that the use of double linkers in the methods of the present invention permits easier regeneration of a detection system for multiple readings by the use of relatively weak aqueous buffer without the need for strong acidic, basic or organic solvents.

In a currently preferred embodiment, a streptavidin/biotin linkage with a short aminocaproic acid chain (conjugate **6** in Figure 3) is used in the construction of the

first linker between a binding partner and a nanoparticle, which is 10 nanometres in size. When a large size of nanoparticle such as a 20 nm bead is used, the first linker should preferably be designed much longer for consideration of easy regeneration on the sensor surface.

In a preferred embodiment, the present invention relates to a new design of optical biosensor-based competitive immunoassays (Figure **1**) particularly surface plasmon resonance (SPR)-based immunoassays for small molecular weight haptens, such as therapeutic and abused drugs, steroids, thyroid hormones, metabolites and pollutants. This SPR-based immunoassay format method comprises the steps:

(a). chemically immobilising hapten (**A**) or hapten conjugate onto the optical biosensor surface through a linker molecule (the second linker) with or without using a hapten attachment intermediate,

(b). mixing a fixed concentration of binding partner (**B**)-(the first linker)-nanoparticle conjugate in buffer with each of a series of standard free solution or a sample hapten solution and incubating for a few minutes,

(c). injecting the above mixture or the remaining binding partner (**B**) in equilibrium solution onto the hapten (**A**) biosensor surfaces, and measuring binding partner (**B**) responses,

(d). injecting regeneration buffer onto the biosensor surface to remove binding partner-(the first linker)-nanoparticle conjugate,

(e). repeating the above experiments (b), (c) and (d) three times or more for reproducibility,

(f). plotting concentrations of free hapten versus average response (RU) of binding partner -(the first linker)-nanoparticle conjugate to provide an assay standard curve from which determining the concentration of unknown sample hapten when using the same method.

With reference to Figure **1**, the currently most preferred embodiment of the invention is now described. design of "dual-linker" and "nanoparticle" is: (1) For hapten conjugate; ovalbumin (protein)---linker (10~30 atoms in length) (thiopropanoic acid with 1~3 aminocaproic acids)---small molecular hapten (progesterone)(Figure **2**); (2) For binding partner conjugate; antibody---long linker (complex of streptavidin/biotin complex plus one aminocaproic acid chain)---gold nanoparticle (10

nanometre)(Figure 3). Based on the above design, a rapid flow-through (BIAcore 2000) and sensitive immunoassay for small molecular hapten (progesterone, MW = 314.47) is achieved. The lowest detection limit (LDL) for the assay is around 0.1 pg/ml or 0.3 pM (3 X 10^{-13} M).

Based on the concept of a dual-linker combined with nanoparticle enhancement, the use of all other variations on the above methods by, for example, including various nanoparticles in different sizes, different types, lengths, and molecular hybridisations of dual linkers fall within the scope of the present invention.

The invention also extends to kits comprising a first and a second moiety with their various attachments as described above in separate containers with or without instructions for their use.

The invention is illustrated by the following non-limiting examples.

EXAMPLE 1

Preparation of 4-Progesterone Acid Derivative (7) and Its Ovalbumin Conjugate (3)

A solution of ε-aminocaproic acid (44.4 mg (0.34 mM) in 200 μL of UHQ water) was added drop-wise to a solution of progesterone 18-atom linker-succinate active ester (*Steroids,* **67**, 2002, 565-572) (83.8 mg (0.11 mM) in 2 mL of dry DMF). 0.5 mL of dry DMF was used to wash out the ε-aminocaproic acid vial. The reaction was stirred over a weekend. The solvent was removed under vacuum and the resultant yellow-tinged oil reconstituted in 100 mL of chloroform and washed with 3x50 mL of distilled water. The solvent was removed under vacuum, and the resultant light brown oil was column separated using a 15:1, 10:1, 5:1, 1:1, 0:1 chloroform:methanol eluent series. The resultant clear, colourless oil was washed with a diethyl ether, n-hexane, chloroform mixture to give waxy white solids (7). Yield: 68.1 mg (80%). R_f = 0.77 (5:1 chloroform:methanol). ^{1}H NMR: δ 0.68 (s, 3H, 18-CH_3), 1.25 (s, 3H, 19-CH_3), 2.14 (s, 3H, 21-CH_3), 2.84 (t, 2H, J=6.8Hz, S-CH_2), 3.71 (d of t, 1H, J=14.7Hz, 6α-H). ^{13}C NMR: δ 13.4 (18-C), 17.6, 18, 18.2 (19-C), 21.2 (11-C), 22.9 (15-C), 23.3,

24.3 (linker C), 25, 25.6, 26, 26.6, 29.2 (linker C), 29.8, 30.5, 30.8, 31.2, 31.8 (21-C), 31.9, 32.1 (6-C), 34.5, 34.7, 34.9, 35.7 (8-C), 36.8 (1-C), 36.9, 38.7, 39.6 (16-C), 39.8, 41.6 (10-C), 44 (13-C), 54.2 (9-C), 56, 63, 63.5 (17-C), 65.9, 171.5 (5-C), 173.8, 176.9, 196 (3-C), 209.5 (20-C), one overlapping peak. Analytical HPLC: 100% pure. 50°C, gradient of 30%B over 5min. then 30 – 80%B over 25min., A = 90:10 dH_2O: MeOH, B = 90:10 MeOH:dH_2O, $pH_{A\&B}$ = 4.2, R_t = 22.1 min. ES-MS: (MeOH, 40 V) 759 $[M+H]^+$, 781 $[M+Na]^+$.

A solution of DCC (17.7 mg in 250 μL dry DMF) was added drop-wise to a stirring solution of above progesterone acid derivative 7 (50 mg in 2mL of dry DMF) and 250 μL of dry DMF used to wash out the vial. A solution of NHS (9.9 mg in 250 μL of dry DMF) was then added drop-wise and a further 250 μL of dry DMF used to wash. 0.5 mL of DMSO was then added to aid dissolution. The reaction was left stirring in the dark overnight. Conjugation to OVA was then done as the same procedure for other conjugates to produce conjugate **3** (*Steroids,* **67,** 2002, 565-572).

EXAMPLE 2

Synthesis of progesterone-4-mercaptopropionamide-ethylthiol (**8**)

Progesterone-4-mercaptopropionyl succinate (*Steroids,* **67,** 2002, 565-572) (100 mg, 0.194 mmol) was dissolved in dry DMF (1mL) and a solution of mercaptoethylamine (44.8mg, 0.581mmol, in 0.5mL dry DMF) was added drop-wise followed by a further 0.5 mL of DMF to wash. The reaction was stirred overnight at room temperature. Solid formed was filtered off and the filtrate solvent was removed in vacuo. The resulting oil was washed with chloroform and the chloroform phase was column separated using $CHCl_3$, 15:1 $CHCl_3$:MeOH, 10:1 $CHCl_3$: MeOH, 5:1 $CHCl_3$: MeOH eluent to yield an oil. Yield: 17.1mg (18%). R_f = 0.52 (15:1 chloroform:methanol). ^1H NMR ($CDCl_3$): δ 0.70 (s, 3H, 18-CH_3), 1.26 (s, 3H, 19-CH_3), 2.15 (s, 3H, 21-CH_3), 2.45 (t, 1H, J = 7Hz), 2.53 (m, 3H), 2.88 (m, 4H, 2 x S-CH_2), 3.62 (m, 2H, CONH-CH_2), 3.73 (d, 1H, J = 14Hz, 6α-H). ^{13}C NMR ($CDCl_3$): δ 13.4 (18-C), 18.1 (19-C), 20.8 (11-C), 23.0 (15-C), 24.3 (16-C), 25.0 (S-CH_2), 25.7 (S-CH_2), 30.5 (7-C), 31.5 (21-C), 32.1 (C-6), 34.0 (2-C), 34.2 (N-CH_2), 34.4 (1-C), 35.7 (8-C), 36.5 (CH_2CO),

38.7 (12-C), 41.6 (10-C), 43.8 (13-C), 54.2 (9-C), 55.8 (14-C), 63.5 (17-C), 129 (4-C), 172 (5-C), 175 (amide C = O), 195 (3-C), 209 (20-C). ES-MS: 476 Da $[M-H]^-$.

EXAMPLE 3

Synthesis of Progesterone-PEG (220)-NH_2 Derivative (**9**)

4-mercapto-progesterone acid (200 mg) was dissolved in DMF (dry, 1mL) and DCC (128 mg in 0.5 mL dry DMF) was added dropwise followed by NHS (71.3 mg in 0.5 mL dry DMF). The reaction was stirred in the dark overnight before filtering off the solid. Mono-PEG-Boc (458.2 mg) was dissolved in dry chloroform (1 mL) and added dropwise to the stirring ester solution. Triethylamine (0.5 mL) was then added and the reaction stirred over the weekend in the dark. The solvent was removed in vacuo and the mixture was separated by column using 15:1 chloroform:methanol eluent to yield yellow oil for amine-protected product (progesterone-PEG-NHBoc). Yield: 169.8 mg (49%). R_f = 0.36 (15:1 chloroform:methanol). ^1H NMR ($CDCl_3$): δ: 0.65 (s, 3H, 18-CH_3), 1.13 (s, 3H, 19-CH_3), 1.41 (s, 9H, Boc CH_3), 2.09 (s, 3H, 21-CH_3), 2.89 (m, 6H, PEG), 3.57 (m, 14H, PEG). ^{13}C NMR ($CDCl_3$) δ: 13.7 (18-CH_3), 18.4 (19-CH_3), 21.5 (11-CH_2), 23.2 (15-CH_2), 24.5 (16-CH_2), 25.3, 26.0 (S-CH_2), 28.7 (Boc CH_3), 29.4, 30.0, 30.9, 31.0 (7-CH_2), 31.7 (21-CH_3), 32.4 (6-CH_2), 34.3, 34.7 (1-CH_2), 34.9, 35.6, 36.7 (17-CH), 37.0, 38.9, 39.1 (12-CH_2), 41.7 (10-C), 44.2 (13-C), 49.1, 54.5 (9-CH), 56.3 (14-CH), 63.7 (17-CH), 69.8 (PEG C-O), 70.0 (PEG C-O), 70.5 (PEG C-O), 70.9 (PEG C-O), 129.0 (4-C), 156.3, 162.8, 171.4 (5-C), 176.2 (carbonyl), 195.7 (3-C), 209.5 (20-C). ES-MS (MeOH): $[M+H]^+$ 722, $[M+Na]^+$ 744.

The final free amine product or progesterone–PEG (220)–NH_2 (**9**) can be easily synthesised from the above Boc-protected compound by deprotection in formic acid (98% pure).

EXAMPLE 4

Synthesis of Progesterone–PEG (220)–Biotin (**10**)

Progesterone–PEG (220)–NH_2 (160 mg) was dissolved in chloroform (1.5 mL, dried over molecular sieves 4A). Biotin active ester (113.8 mg in 1mL of dry DMF with warming) was added dropwise to the stirring progesterone–PEG (220)–NH_2 solution. The solution was stirred in the dark for two hours before addition of triethylamine (0.5 mL) after which it was left stirring over the weekend. A solid initially forms but by the end of the reaction it has gone. The solvent was removed in vacuo and then column separated using 10:1 chloroform:methanol and 5:1 chloroform:methanol eluent. Yield (**10**): 95.5mg (44%). R_f = 0.70 (5:1 chloroform:methanol). ^{1}H NMR ($CDCl_3$): δ 0.70 (s, 3H, 18-CH_3), 1.25 (s, 3H, 19-CH_3), 1.72 (m, biotin), 1.80 (m, biotin), 2.14 (s, 3H, 21-CH_3), 2.95 (m, 5H, PEG), 3.20 (d, 1H, biotin), 3.37 (m, 2H, PEG), 3.62 (m, 13H, PEG), 4.36 and 4.54 (d of t, 2H, biotin), 5.16 and 5.23 (d, 1H, biotin). ^{13}C NMR δ. ES-MS: 848.1 $[M+H]^+$, 870.1 $[M+Na]^+$.

EXAMPLE 5

4-Mercapto-Estrone Acid (**11**)

Estrone (400 mg, 1.48 mmol) was dissolved in dry ethanol (10 mL) and acetone (10 mL). *N*-bromosuccinimide (263.3 mg, 1.48 mmol) was added to the vigorously stirring solution and the solution stirred at room temperature for 24 hours. The white solid formed was filtered off and washed with ethanol (174.5 mg, 34%). Removal of the filtrate solvent and recrystalisation of the resultant solid as 4-bromoestrone increases the yield to 43%. Mp 254 °C (literature 281-282 °C); R_f = 0.23 (4:1 petroleum spirit 60-80 °C: ethyl acetate); ^{1}H NMR 0.90 (3H, s), 0.90 (1H, s), 1.26 – 2.96 (m), 5.37 (1H, s), 6.86 (1H, d, *J*= 8.6Hz), 7.18 (1H, d, *J*= 8.6Hz); ES-MS *m/z*.

4-bromoestrone (150 mg, 0.43 mmol) was dissolved in dry methanol (20 mL) and potassium hydroxide (15 mL, 23.4 $mgmL^{-1}$ in dry methanol) was added whilst stirring, followed by 3-mercaptopropionic acid (424.8 μL) and refluxed under dry conditions for 24 hours. The sample was then cooled and solvent removed. The sample was reconstituted in distilled water (25 mL) and extracted with ethyl acetate (2 x 12.5 mL, 1 x 25 mL). The solvent was removed and the sample recrystallized from chloroform to provide pure 4-mercapto-estrone acid **11** (42.6 mg, 27%): Mp 108-112

°C; R_f = 0.12 (15:1 chloroform:methanol); ^{1}H NMR 0.87 (3H, s, 18-CH_3), 1.23-3 (17H, m, estrone fine structure), 3.04 (2H, t, J = 1.9, S-CH_2), 6.50 (1H, d J = 8.7, C-2), 6.80 (1H, d, J = 9.0, C-1); ^{13}C NMR 17.5, 23.4, 25.5, 28, 30, 30.7, 34.5, 35, 39.5, 41.5, 42.3, 48.1, 54.2, 117, 118.8, 119.2, 123.5, 125.4, 129, 159, 178.4; ES-MS: *m/z* 374.5 $[M+H]^+$, 397.5 $[M+Na]^+$.

EXAMPLE 6

4-Mercaptol-Estradiol Acid (**12**)

4-bromoestradiol (200 mg) was dissolved in dry methanol (20 mL). Methanolic potassium hydroxide (20 mL, 7.8 $mgmL^{-1}$) was added followed by 3-mercaptopropionic acid (550 μL). The solution was refluxed under dry conditions for 24 hours in the dark. The solvent was removed and the sample reconstituted in distilled water (50 mL). The aqueous phase was washed with ethyl acetate (2 x 25 mL, 1 x 50 mL). The aqueous phase had its pH adjusted to 2.5, which crashed a white solid out of solution. The solid was separated by centrifugation and washed three times with water and then dried to yield a white solid **12** (103.4 mg, 46%). mp 78-84 °C; R_f = 0.46 (ethyl acetate); ^{1}H NMR 0.81 (3H, s, 18-CH_3), 1.38-2.3 (m, estradiol fine structure), 2.75 (3H, t, J= 4.6, 17-CH), 2.81 (2H, t, J= 4.5, S-CH_2), 6.89 (1H, d, J= 6.3, 2-H), 7.22 (1H, d, J = 6.7Hz, 3-H); ^{13}C NMR 10.4 (18-CH_3), 14.2, 21.2, 21.4, 22.8, 23.1, 24.0, 25.4, 26.8, 29.1 (S-CH_2), 29.8, 30.2, 31.0, 33.8, 34.2, 37.1, 50.9 (17-CH), 74.6, 90.5, 171.5 (3-C), 194 (COOH); ES-MS *m/z* 399.1 $[M+H]^+$, 406.8 $[M+OMe]^-$.

EXAMPLE 7

4-Estradiol–PEG (220) –NH_2 (**13**)

4-Estradiol acid (80 mg, 0.201 mmol) was dissolved in dry DMF (1 mL) and DCC (53.9 mg in 0.5 mL of dry DMF, 0.2613 mmol) was added dropwise to the vigorously stirring solution followed by NHS (30.1 mg in 0.5 mL of dry DMF, 0.2613 mmol). The solution was stirred overnight at room temperature in the dark. A white solid

formed within 30min of addition. The solid was filtered off and the solvent removed. The sample was then column separated using 15:1 chloroform:methanol, 10:1 chloroform:methanol and 5:1 chloroform:methanol. The pure product (4-estradiol succinimidyl ester) was isolated as a white solid (44.0 mg, 44%). Mp = 149-156 °C. R_f = 0.48 (10:1 chloroform methanol). ^{1}H NMR: δ 0.82 (3H, s, 18-CH_3), 1.05-2 (m, estradiol fine structure), 2.73 (t, 17 CH), 2.90 (2H, t), 2.97 (4H, s, NHS protons), 8.03 (2H, s, aromatic ring); ^{13}C NMR 25.2 (CH_2), 25.7 (CH_2), 25.9 (CH_2), 27.3 (CH_2), 29.9 (S-CH_2), 30.0 (CH_2), 31.5 (18-CH_3), 31.9 (CH_2), 32.7 (CH_2), 33.5 (CH_2), 34.0 (CH_2), 34.3 (CH_2), 34.5 (succinate CO), 35.0 (succinate CO), 37.0 (CH), 49.8 (CH), 51.0 (17-CH), 52.2 (CH), 154.1 (C), 158.0 (C), 163.3 (CH), 169.2 (C), 172.5 (CH), 175.2 (3-C), 175.4 (ester); ES-MS *m/z* 471.6 $[M+H]^+$.

The above synthesised 4-estradiol succinimidyl ester (50 mg, 0.106 mmol) was dissolved in dry DMF (1 mL) and stirred rapidly whilst mono-Boc protected PEG (220) (102.6 mg, 0.372 mmol in chloroform, 0.5 mL) was added drop-wise followed by triethylamine (0.25 mL). The solution was then stirred over the weekend at room temperature in the dark. The solvent was then removed and the resulting oil column separated using 15:1 chloroform:methanol, 10:1 chloroform:methanol, 5:1 chloroform:methanol eluent sequence, yielding pure compound [4-estradiol-PEG (220)-NHBoc] as a clear, colourless oil (62.3 mg, 0.098 mmol, 93% yield). R_f = 0.36 (10:1 chloroform: methanol). ^{1}H NMR: δ 1.24 (2H, t, J = 7.0), 1.44 (9H, s, Boc CH_3), 1.79 (5H, m), 2.59 (2H, t, J = 7.4), 2.74 (3H, t, J= 6.2), 2.98 (5H, m), 3.37 (2H, m), 3.60 (14H, m), 5.06 (1H, s), 6.82 (1H, s, aromatic estradiol); ^{13}C NMR: 18.4 (estradiol CH_3), 26.4, 27.2, 28.5, 28.7 (Boc CH_3), 29.7, 33.2, 33.3, 33.8, 34.0, 34.3, 34.6, 36.2, 36.5, 38.0, 38.4, 50.6, 52.0, 58.4, 69.4, 69.9 (PEG C-O) 70.1 (PEG C-O), 70.2 (PEG C-O), 70.5 (PEG C-O), 70.5 (PEG C-O), 79.3 (17-CH), 100.3, 102.8, 109.8, 127.6, 139.1, 156.3, 171.4 (CH), 171.7, 175.1 (Boc carbonyl), 181.1 (mercaptopropionate carbonyl); ES-MS (MeOH, 45V) 535.4 $[M\text{-Boc}+H]^+$, 557.4 $[M\text{-Boc}+Na]^+$, 652.4 $[M+NH_4]^+$, 670.4 $[M+H_2O+NH_4]^+$.

The final free amine product or 4-estradiol–PEG (220)–NH_2 (**13**) can be easily synthesised from the above Boc-protected compound by deprotection in formic acid (98% pure).

4-Estradiol–PEG (900)– NH_2 (**14**)

Polyethylene glycol (900) [O, O'-Bis-(2-aminopropyl)polypropylene glycol-block-polyethylene glycol-block polypropylene glycol, Fluka 14527] (2 g, approx. 2.222 mmol) was dissolved in dry methanol (20 mL) and dry triethylamine (1 mL) was then added. Boc reagent (0.4856 g, 2.222 mmol) was dissolved in dry methanol (10 mL) and added drop-wise to the above rapidly stirring PEG solution over ~ 20 min using a syringe and septum. The solution was then left to rapidly stir overnight at room temperature. The solvent was then removed and the sample was separated by a column using 32:1:1, 32:2:1, 32:4:1, 16:4:1 dichloromethane:methanol:acetic acid eluent to yield mono-protected PEG (900) as a clear colourless semi-solid (911.4 mg, 41% yield). R_f = 0.53 (32:2:1 dichloromethane:methanol:acetic acid). ^{1}H NMR: δ 1.13 (s, 8H), 1.27 (s, 3H), 1.44 (s, 9H, Boc CH_3), 2.00 (s, 6H), 3.45 (s, 7H), 3.65 (s, 65H, ethylene protons); ^{13}C NMR: 15.0, 15.3, 15.4, 16.1, 16.8, 16.9, 17.0, 17.9, 18.8, 22.5, 28.4 (Boc CH_3), 46.6, 47.1, 47.2, 48.4, 70.3 (cluster), 72.5, 72.6, 74.4, 74.9, 75.2, 75.5, 76.2, 155.5, 176.1 (Boc-carbonyl). ES-MS: (MeOH 40V) multiple peaks corresponding to different n-values of the PEG chain.

4-Estradiol succinimidyl ester (50mg, 0.106mmol) was dissolved in dry DMF (1 mL) and stirred rapidly whilst mono-Boc PEG (900) (371.7 mg, approx. 0.372 mmol dissolved in 5:1 chloroform:methanol, 3 mL) was added drop-wise followed by triethylamine (0.5 mL). The solution was stirred at room temperature over the weekend in the dark. The solvent was then removed and the resulting orange oil column separated using 15:1 chloroform: methanol, 10:1 chloroform: methanol, 5:1 chloroform: methanol eluent to yield pure protected product [4-estradiol-PEG (900)-NHBoc] as a clear, colourless oil (39.5 mg, 0.029 mmol, 27% yield). R_f = 0.73 (5:1 chloroform:methanol). ^{1}H NMR: δ 1.14 (14H, m), 1.44 (9H, s, Boc CH_3), 2.58 (2H, t, J= 7.1), 2.73 (3H, t, J = 7.0), 2.97 (6H, m), 3.47 (m), 4.91 (1H, s), 6.75 (1H, t of d, J = 34.9, J = 7.9); ^{13}C NMR: 16.7, 17.1, 17.6, 18.0, 28.5 (Boc CH_3), 29.7, 34.1, 34.3, 36.2, 45.1, 45.5, 70.6 (PEG C-O), 71.9 (PEG C-O), 72.1, 72.4, 72.6, 73.4, 74.0, 74.5, 75.1, 75.3, 75.6, 75.9, 126, 128, 130, 155.7, 164, 170.8, 174.4. ES-MS: (MeOH, 40V) multiple peaks from range of PEG chain n-values.

The synthesis of final 4-estradiol–PEG (900)–NH_2 (**14**) is carried out in the same procedure as for 4-estradiol–PEG (220)–NH_2 (**13**) in formic acid (98% pure).

EXAMPLE 8

Dopamine 5-Mercaptopropanoic Acid (**15**)

Dopamine (400 mg, 2.12 mmol) was dissolved in dry methanol (30 mL) and *N*-hydroxysuccinimide (375.2 mg, 2.12 mmol) was added and the solution stirred at room temperature in the dark for 24 hours. The solution then had the solvent removed and was reconstituted in distilled water (50 mL) and washed with chloroform (2 x 25 mL, 1 x 50 mL) and the solvent removed from the aqueous phase. The sample was reconstituted in methanol and decoloured thoroughly with activated charcoal. The solvent was then removed to yield 5-bromo-dopaminean as an off-white semi-solid (239.5 mg, 49%). $R_f = 0.54$ (40:1 methanol:acetic acid), ^{1}H NMR 2.94 (2H, t, $J = 7.2$ NH_2-CH_2), 3.17 (2H, t, $J = 6.9$ Ar-CH_2), 6.74 (1H, m, 2-CH), 6.92 (1H, m, 5-CH); ^{13}C NMR 31.0 (Ar-C), 31.85 (Ar-C), 39.4 (C-NH_2), 40.5 (C-NH_2), 115.7 (2-C), 116.5 (5-C), 116.6 (6-C), 117.0 (3-C), 118.0 (4-C), 124.2 (1-C); ES-MS *m/z* 233 isotope pattern $[M+H]^+$.

The above synthesised 5-bromo-dopamine (100 mg, 0.429 mmol) was dissolved in dry methanol (5 mL) and methanolic KOH was added (11.8 $mgmL^{-1}$, 5 mL) with vigorous stirring. 3-mercaptopropionic acid (113.7 μL) was added and the reaction refluxed under dry conditions for 24 hours. The solvent was then removed and the resultant semi-solid constituted in distilled water (25 mL). The aqueous phase was washed with ethyl acetate (2 x 12.5 mL, 1 x 25 mL) and the aqueous phase acidified to pH = 1. The solvent was removed from the aqueous phase to yield a yellow-white semi-solid (250.6 mg), which was then passed through a short silica column using 40:1 methanol:acetic acid eluent to yield pure product **15** as a white solid (44.1 mg, 40% yield). Mp= 292-298 °C, $R_f = 0.55$ (40:1 methanol: acetic acid), ^{1}H NMR: δ 2.44 (2H, t, $J = 9.5$, CH_2-COOH), 2.77 (2H, t, $J = 9.7$, CH_2-S), 2.54-2.88 (2H, m, CH_2-Ar), 3.19-3.57 (m, CH_2-NH_2); ^{13}C NMR: 23.0 (S-CH_2), 23.7 (CH_2-COOH), 34.7

(CH_2-Ar), 36.9 (CH_2-NH_2), 117.3 (C-2, C-5), 122.2 (C-1), 125.4 (C-6), 136.8 (C-3), 143.2 (C-4), 170.3 (acid); ES-MS: *m/z* 255.2 $[M-H]^-$, 279.2 $[M+Na-2H]^-$, 211.9 [M-catechol chain – $H]^+$.

EXAMPLE 9

Serotonin 4-Mercaptopropanoic Acid (**15**)

Serotonin (50 mg, 0.129 mmol as creatinine sulfate) was partially dissolved in dry methanol (5 mL). *N*-bromosuccinimide (23.0 mg, 0.129 mmol in 5 mL of dry methanol) was added and the mixture refluxed for 11 hours. The solvent was then removed and the sample washed with ethyl acetate. The sample was then constituted in methanol and activated carbon used to de-colour it. The resulting clear, colourless filtrate had the solvent removed to yield 4-bromo-serotonin as a slightly brown semi-solid. Mp = 248-249 °C (tentative), R_f = 0.37 (MeOH), ^{1}H NMR: δ 3.25 (2H, t, CH_2-Ar), 3.67 (2H, CH_2-NH_2), 6.90, 7.33 (3H, m, aryl protons), ^{13}C NMR: 33.8 (CH_2-Ar), 39.7 (C-NH_2), 102.5 (indole), 111.9 (C-Br), 112.5 (fused), 112.9 (fused), 117 (C-OH), 123.5 (*o*-OH), 125 (*o*-Br). ES-MS: *m/z*.

The synthesis of serotonin 4-mercaptopropanoic acid **16** from the above 4-bromo-serotonin is performed as the similar procedure to the preparation of dopamine 5-mercaptopropanoic acid **15** from its bromo-precursor.

EXAMPLE 10

Biotination of monoclonal *anti*-progesterone antibody (Figure 3)

Biotinyl-*N*-ε-aminocaproyl-*N*-hydroxysuccinimide ester (***BcapNHS***) was dissolved in dry DMF (5 mg/ml), and the monoclonal *anti*-progesterone antibody (100 µl) was dissolved into 0.1 *M* $NaHCO_3$ (1 ml). Add the ***BcapNHS*** solution in DMF (50 µl) to the above antibody solution in $NaHCO_3$ (1 ml); the solution was allowed to stand at room temperature for 2 hours without stirring.

The solution was then dialyzed overnight against 0.15 *M* NaCl (1 L) with several changes (> 4 times); the last dialysis is performed against PBS/T (1 L) for at least 4 hours. Finally, the biotinylated antibody was further purified by passing through a PD-10 column to give 3.5 ml of pure antibody solution, which is stored at – 20 °C for future uses.

EXAMPLE 11

Direct antibody-binding performance on the biosensor surface

Immobilisations

Immobilization of progesterone-linker (11 ~ 25 atoms linker)-OVA conjugates onto biosensor surfaces (activated CM-5 sensor chip) was done manually aiming for a minimum immobilisation of 2000RU. Progesterone-linker (11-atoms)-OVA conjugate was immobilised at pH 3.5 and progesterone-linker (25-atoms)-OVA conjugate at pH 4.0. Flow rates were 5μL min^{-1} and 2000 RU or greater was achieved in both cases. Final immobilisations were 2524 or 2208 RU for the above two conjugates respectively. The chip had a solution of OVA (5 μgmL^{-1} in running buffer) passed over the surface to help to stabilise it (10 min. at 25 μLmin^{-1}). Immobilisation buffers were 10 mM sodium formate as previously (*Steroids,* **67,** 2002, 565-572).

Binding Performance with Unmodified Antibody

Monoclonal anti-progesterone (un-modified) was passed over the surface to assess its binding (100 μgmL^{-1} in running buffer, 3 min. injection at 20 μLmin^{-1}). This resulted in a binding of 654 RU for conjugate with 11-atoms linker, and 447 RU for the conjugate with 25-atoms linker. Regeneration was effected with 50 mM glycine buffer pH = 1.5 (two pulses of 75 μL at 50 μLmin^{-1} flow rate) and this were adequate for complete baseline return.

Binding Performance with Biotinated Antibody

Biotinylated monoclonal antibody was then passed over the surface (100 μgmL^{-1} in running buffer, 3 min. injection at 20 μLmin^{-1}) and gave a binding of 406 or 142 RU for two conjugates respectively. This result indicates that the presence of biotin-linker

units on the antibody has a significant effect on the degree of binding causing a 35% reduction for the conjugate having a 11-atoms linker, and a 60% reduction for the conjugate having a 25-atoms linker.

Binding Performance *with Antibody-Nanogold Particle Conjugate*

Biotinylated monoclonal antibody (100 μgmL^{-1} in running buffer, 100 µL) was mixed 1:1 with 10 nm colloidal gold–streptavidin conjugate (Sigma S9059) and vortexed, and then incubated at room temperature for 10 min before injection (120 µL, 20 $\mu Lmin^{-1}$). The resulting binding was 667 RU for the conjugate having an 11-atoms linker and 257 RU for the conjugate having a 25-atoms linker. This represents a signal enhancement of 64% or 82% for both conjugates respectively. Regeneration was again done using 50 mM glycine pH 1.5 as before and found to give complete return to baseline.

In order to determine the best antibody/gold volume ratio to use for competitive assay development, various ratios were optimised according to their antibody binding responses. The biotinylated monoclonal *anti*-progesterone was set at a concentration of 100 μgmL^{-1}. The ratios tested were 1:1 (80µL mAb:80µL gold), 1.67:1 (100µL:60µL), 3:1 (120µL:40µL), 7:1 (140µL:20µL) and 15:1 (150µL:10µL). The same testing was then done but with running buffer instead of gold colloid to determine the degree of gold signal enhancement at each ratio. The results are summarised below in Figure 7 for the conjugate having an 11-atoms linker, and Figure 8 for the conjugate with a 25-atoms linker.

The results clearly show that as the monoclonal antibody volume is increased without gold labelling, one observes an increase in response up until a ratio of 3:1 antibody: buffer after which it begins to decrease slowly. This pattern is seen for both conjugates the difference being the conjugate with an 25-atoms linker has much lower overall response than the other conjugate (11-atoms linker).

When considering the monoclonal antibody: gold colloid ratio, signal continues to increase up to a ratio of 7:1 mAb:gold though flattens out at the end and from 7:1 to

15:1 a slight decrease in response is observed for both conjugates. Once again the 4-3 response is much lower than that for 4-1.

The degree of gold colloid signal enhancement (expressed in absolute terms or as a percentage) is seen to peak at around 1.5:1 mAb:gold ratio and drop again until 3:1 after which a modest increase is observed up to 7:1. This suggests that gold enhancement is maximal at around 1.5:1 ratio and is less significant at higher antibody:gold ratios. Based on the signals obtained from the ratios above, the ratio giving largest overall signal considering both conjugates was selected as the ratio to use in development of a progesterone assay curve. The ratio selected was 7:1 mAb:gold.

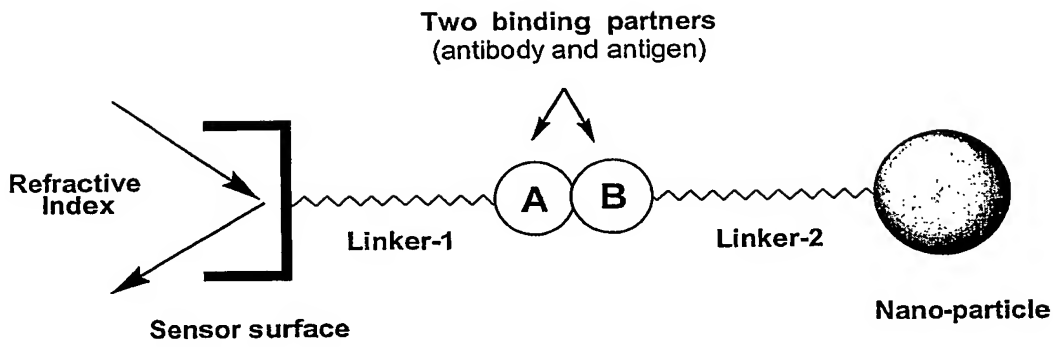
EXAMPLE 12

Competitive Progesterone Immunoassay Using Progesterone-OVA Conjugate Surface and Antibody-Nanogold Conjugate as Flow Immunoreactant

A series of standard progesterone solutions were prepared in HBS buffer, at concentrations ranging from 0 to 1 μg/ml. Each sample (100 μl) was incubated with an equal volume (100 μl) of mixture of mAb (100 μgmL^{-1}):streptavidin/nanogold (10 nm) (7:1), incubating for 5min at 25 ^0C, and the resulting mixture (120 μl) passed over the chip surfaces for 6 minutes at a flow rate of 10 $\mu lmin^{-1}$. The regeneration of sensor surfaces was performed by two glycine buffer (50 mM, pH 1.5, 50 $\mu lmin^{-1}$, 2 min) pulses. The same procedure was carried out three times for each concentration.

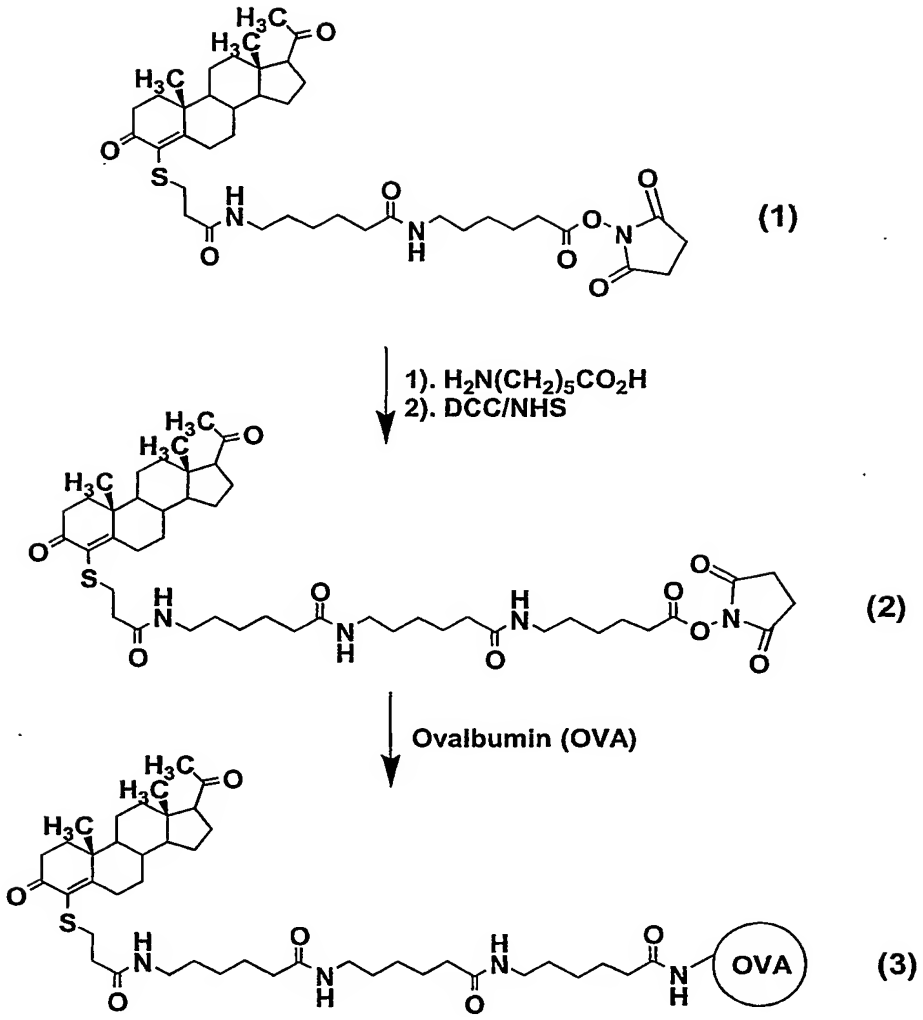
A plot of concentrations of free progesterone versus percentage (%) bound of RU relative to zero progesterone concentration provides two standard curves for two progesterone-OVA conjugates. The standard curve for progesterone-OVA conjugate with a 25-atoms linker is shown in Figure 4. The assays for both conjugates demonstrate a very broad detection region from 1 μgmL^{-1} to < 0.1 $pgmL^{-1}$. The lowest detection limit is assessed as < 0.1 $pgmL^{-1}$ by both the 90% bound and zero – three standard deviations method, and the 50% bound values are both given in Figure 9.

Figure 1



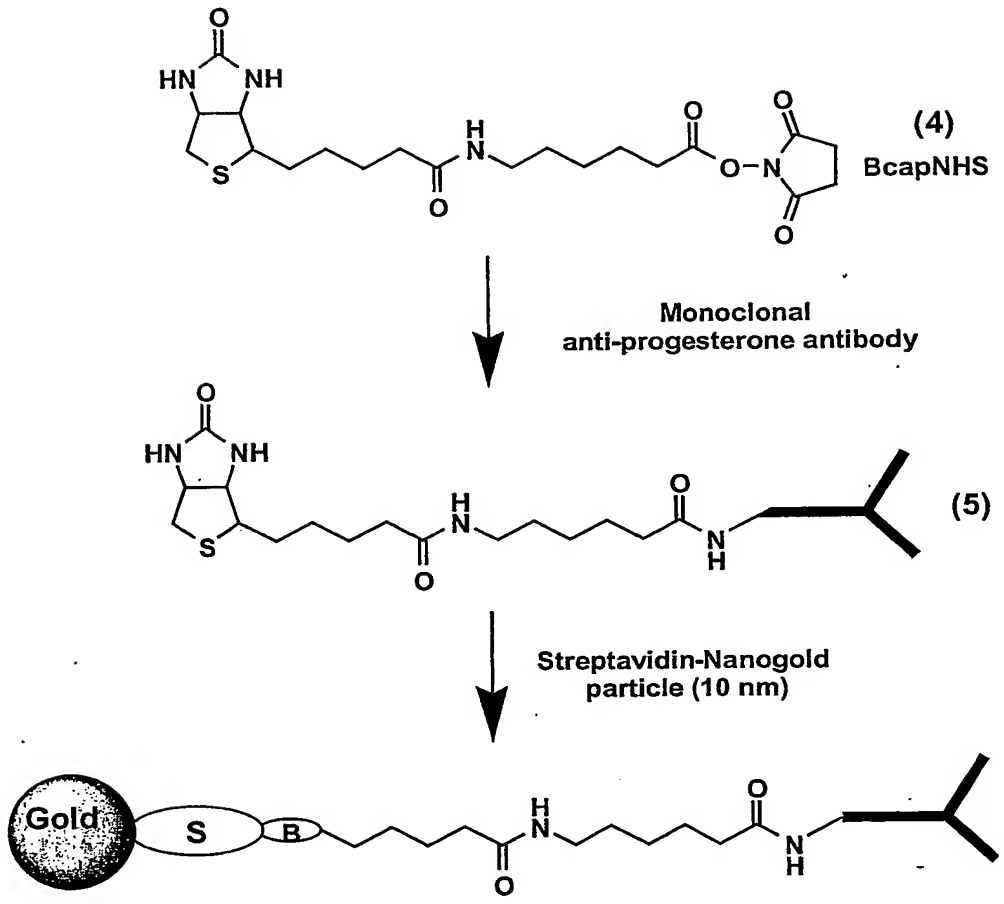
A new optical biosensor-based immunoassay design

Figure 2



Progesterone-OVA conjugate with a 25-atoms linker

Figure 3



Antibody-linker 2-nanogold conjugate (6)

Figure 4

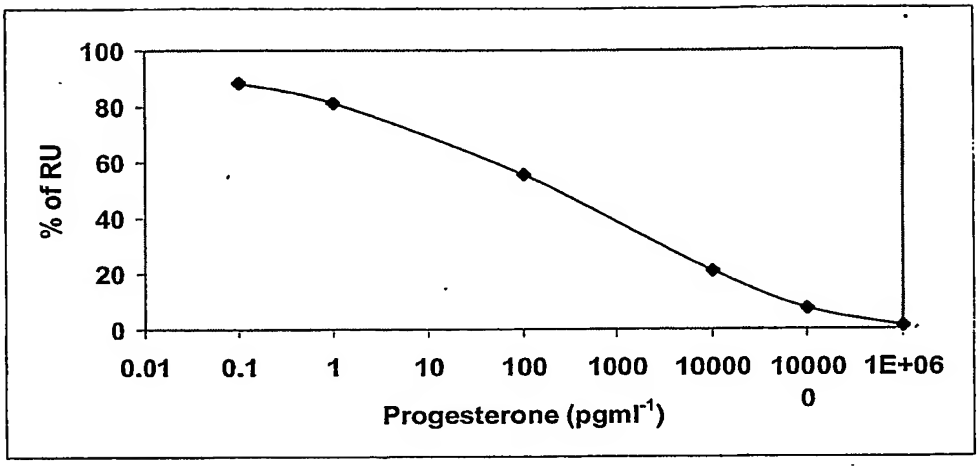
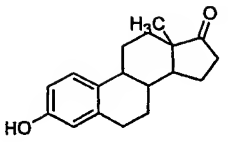
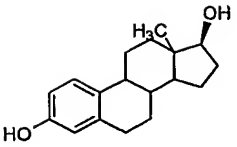

100
80
60
40
20
0
% of RU
0.01
0.1
1
10
100
1000
10000
100000
1E+06
Progesterone (pgml-1)

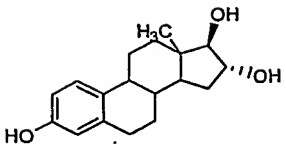
Figure 5



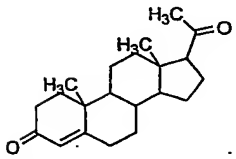
Estrone



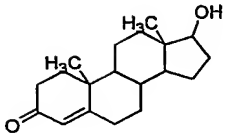
Estradiol



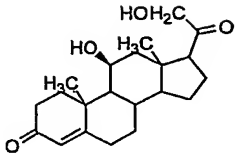
Estriol



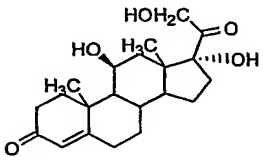
Progesterone



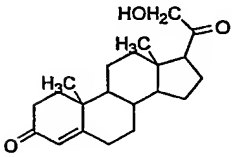
Testosterone



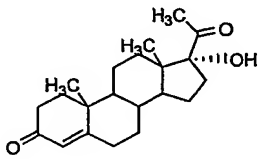
Corticosterone



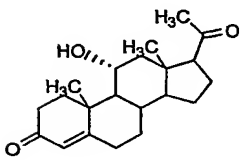
Hydrocortisone



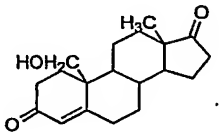
21-Hydroxyprogesterone



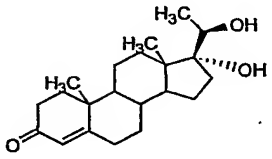
17α-Hydroxyprogesterone



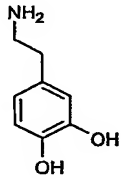
11α-Hydroxyprogesterone



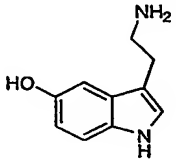
19-Hydroxy-4-androsterone-3,17-dione



17α, 20β-Dihydroxy-4-pregnen-3-one



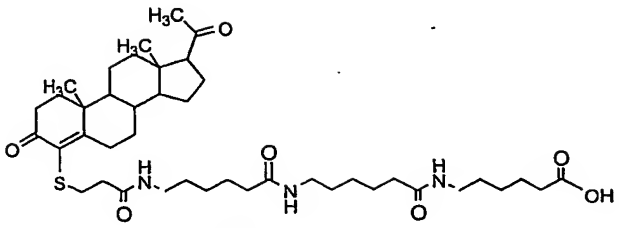
Dopamine



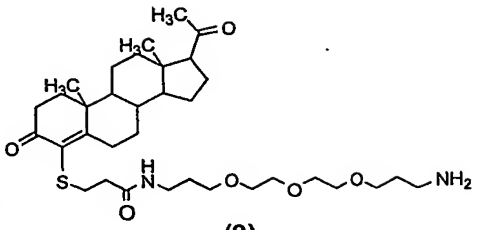
Serotonin

Figure 6

Progesterone derivatives



(7)



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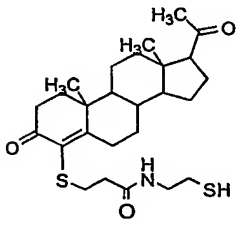

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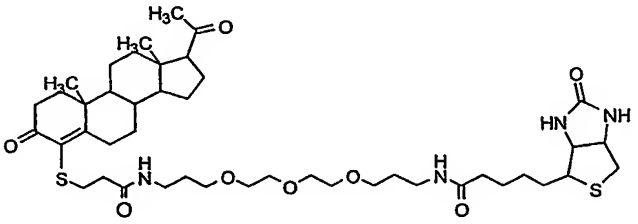
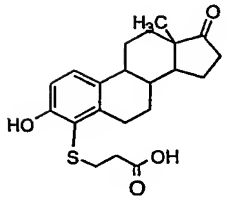


(10)

Estrogen derivatives



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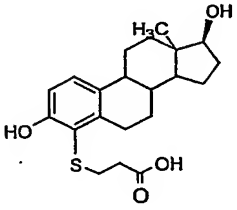

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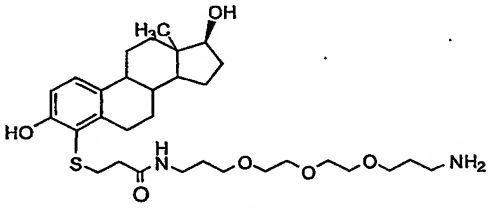

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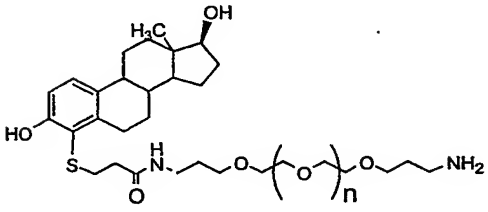
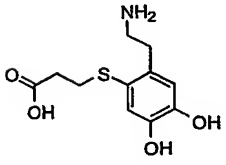


(14)

Neurotransmitter derivatives



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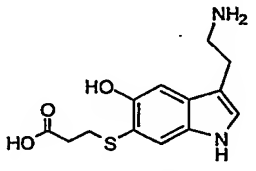

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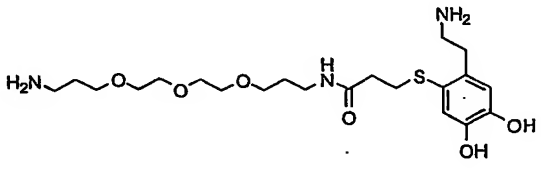

(17)

Figure 7

Volume Ratio mAb:gold	1	1.67	3	7	15
mAb Only	497.9		802.3	731.9	
mAb Gold	796.3	890.5	929	957.1	893.5
Enhancement	298.4		126.7	225.2	
% Enhancement	60		16	31	

Figure 8

Volume Ratio mAb:gold	1	1.67	3	7	15
mAb Only	184.4		292.9	266.8	
mAb Gold	329.6	352.6	371.6	370.2	330.2
Enhancement	145.2		78.7	103.4	
% Enhancement	79		27	39	

Figure 9

Conjugate	50% Bound (pgmL-1)	Detection Limit (pgmL-1)
11-atoms linker	1300	0.1
25 atoms linker	89	0.1

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